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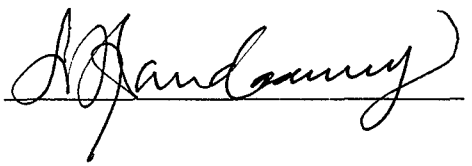

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Growth factor-receptor tyrosine kinases (RTK's) play a central role in the coordination of cell growth, differentiation and other activities in multicellular organism. Molecular lesions in and/or aberrant expression of RTK's can lead to cancer. There is a particularly strong correlation between the erbB2 (neu/HER-2) receptor and breast cancer. The cell-surface location of the erbB2 receptor makes it an obvious target for novel therapies. The purpose of this research is to gain insight into the structure of the extracellular domain of this receptor and its mode of activation in order to aid in the development of new antagonists. ErbB2 is one of a family of 4 RTK's which also includes the epidermal growth factor receptor (erbB1/EGFR). The first step in the stimulation of a response by a growth factor is dimerization of the receptor upon binding of the cognate ligand. We have crystals of the EGF induced homodimer of the soluble extracellular domain of erbB1. Determination of this structure is in progress. This structure will provide insight into the specific molecular events that drive erbB receptor dimerization. A key question is whether the dimerization is mediated only by receptor-receptor contacts, or whether the ligand is bivalent in nature, simultaneously contacting both receptor molecules in the dimer.

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# Introduction

Growth factor-receptor tyrosine kinases (RTK's) play a central role in the coordination of cell growth, differentiation and other activities in multicellular organism[1]. Molecular lesions in and/or aberrant expression of RTK's can lead to cancer. There is a particularly strong correlation between the erbB2 (neu/HER-2) receptor and breast cancer[2-5]. The cell-surface location of the erbB2 receptor makes it an obvious target for novel therapies. The purpose of this research is to gain insight into the structure of the extracellular domain of this receptor and its mode of activation in order to aid in the development of new antagonists.

ErbB2 is one of a family of 4 RTK's that also includes the epidermal growth factor receptor (ErbB1, Figure 1). The first step in the stimulation of a response by a growth factor is dimerization of the receptor upon binding of the cognate ligand leading to activation of the intracellular kinase domain[6-7]. Autophosphorylation on specific tyrosines in the RTK leads to the recruitment of downstream signaling molecules through the interaction with their Src homology 2 (SH2) domains[8]. The erbB ligands can be separated into 3 distinct classes: the EGF agonists (which bind only erbB1), the neuregulins (which bind erbB3 and erbB4), and the bispecific ligands such as betacellulin[9]. The erbB ligands contain an EGF-like domain of 50-90 amino acids that, although in some cases only a small portion of the native ligand, is sufficient for its biological function.

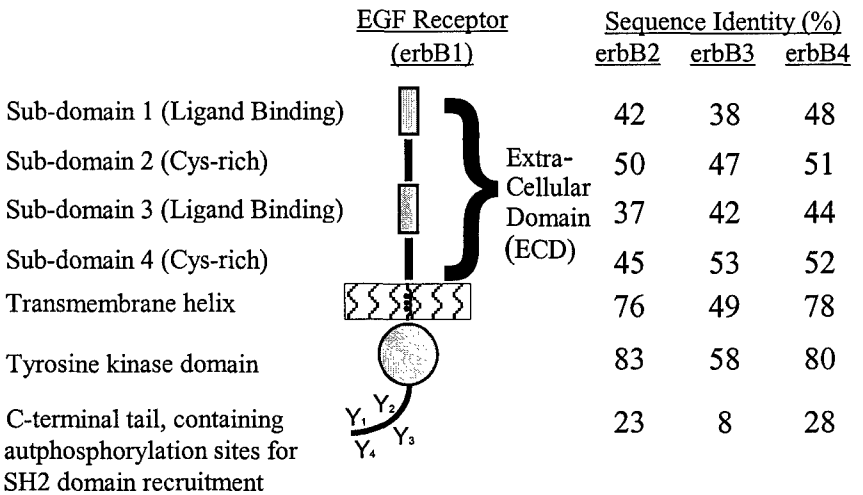


**Figure 1. The erbB family of RTK's.**

The domain structure of the EGF receptor (erbB1) is shown, including the two ligand-binding subdomains (1 and 3) within the ECD. Amino acid sequence identities between the family members are given for each domain and subdomain. While the kinase domain is most well conserved, the C-terminal region that contains the specific SH2 domain-recruitment sequences, is most divergent

The ultimate goal of my research is to determine by X-ray crystallography how EGF induces dimerization of its receptor, and to investigate mechanistic differences between ligand-induced erbB receptor homo- and hetero-dimerization. A detailed structural understanding of receptor activation will provide the basis for designing novel approaches for reversing inappropriate activation in human cancers. I hope, for example, that this will lead to the development of the next generation of erbB2-directed breast cancer treatments.

This report covers progress over the past 3 years during which time I was funded by this award While I have not yet solved the structure, I have made sufficient progress to secure further funding that will allow me to continue working towards a structural understanding of ligand induced activation of erbB receptors.

## Body of Final Report

### *Task 1.* Obtain purified receptor extracellular domains (months 1 - 18)

Two insect cell expression systems are now being used to produce the soluble ectodomains of the erbB receptors (s-erbBs). The first utilizes recombinant baculoviruses and the purification of s-erbBs expressed from this system has been published[11]. More recently the *Drosophila* Expression System (Invitrogen) has been employed to generate stably transfected Schneider 2 cells that express and secrete the ectodomains of s-erbB1, s-erbB2 and s-erbB4.

For the first system, recombinant baculoviruses were generated that allow the secretion from insect cells of the full length extracellular domains of erbB1, 2, 3 and 4 (s-erbBs). C-terminal cleavable His-tags are incorporated to facilitate the first purification step using Ni-affinity chromatography. Production and purification of these proteins is routine with yields ranging from 0.3 mg/L (s-erbB2) to 1.5 mg/L (s-erbB1). Typically 5 - 10 liters of a suspension culture of Sf9 insect cells are grown, in Sf900-II medium (Gibco/BRL), to a density of $2.5 \times 10^6$ cells/ml, and infected at a multiplicity of infection (MOI) of about 5 with freshly amplified, high titer virus solution. Cultures are incubated for a further 96 hours. The clarified conditioned medium is diafiltrated against 3.5 volumes of 25 mM Tris, 150 mM NaCl, pH 8.0 (buffer A), using a Millipore Prep/Scale-TFF 30 kDa cartridge and concentrated to approximately 300 ml prior to loading onto a 5 ml Ni-NTA Superflow column (Qiagen). After extensive washing with buffer A, the column is washed sequentially with 2 column volumes of buffer A containing 30, 50, 75, 100 and 300mM imidazole, pH 8.0. Typically the majority of the protein elutes in the 75 and 100 mM fractions. Fractions are concentrated in an Centricon 30 (Amicon) and loaded on a Pharmacia Superose 6 gel filtration column. At this stage the s-erbBs are greater than 95% pure as assessed by SDS-PAGE analysis. For crystallization, s-erbB1 is further purified by cation exchange chromatography. Fractions from the gel filtration column are pulled, diluted 1.5 fold with 50 mM MES pH 6.0 and loaded on to a BioRad S2 sulfopropyl cation exchange column, pre-equilibrated with 25 mM MES pH 6.0. A gradient in NaCl is developed and the protein elutes at about 200 mM NaCl. The purification utilizes no harsh treatments such as those used to elute protein from antibody affinity columns, which we feel is important in generating material suitable for crystallization. Purified s-erbBs are buffer exchanged into 25 mM Hepes, 100 mM NaCl, pH 8.0, concentrated to between 20 and 100 μM, and stored at 4° C. Concentrations are determined by absorbance at 280 nM using the calculated extinction coefficients from number of Trp and Tyr in each protein.

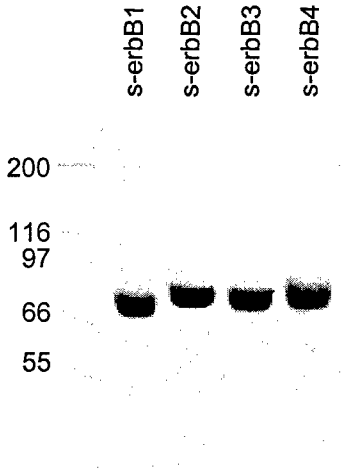


**Figure 2 Purified s-erbB receptors**

SDS-PAGE (7.5% acrylamide) of purified s-ERBB1(s-erbB1), s-erbB2, s-erbB3, and s-erbB4 protein produced from Sf9 cells. Each protein was loaded at 1 mg/ml (10 μl samples), and the gel stained with Coomassie blue.

Recombinant baculoviruses have also been generated to express the isolated ligand binding domain 3 from each of the four receptors. C-terminal His tags are incorporated to aid in purification of this 30 kDa domain. Domain 3 from erbB1 (s-erbB1dom3) has been purified, using a procedure similar to that described above for purification of the full length receptor except that a 10 kDa molecular weight cut off cartridge, and Pharmacia Superose 12 column are employed, as is required for this much smaller (30 kDa) protein. The yield of s-erbB1dom3, about 0.2 mg/L, is lower than for full length s-erbB1. The expressed protein is also less homogeneous. The expressed protein migrates as a doublet on SDS-PAGE. as visualized by Coomassie blue staining and Western blot with antibody to the C-terminal His-tag. These two species can be separated

by cation exchange chromatography but the yield at this step is low. Attempts were made to improve the homogeneity of s-erbB1dom3 by enzymatic deglycosylation using endoglycosidase H. The protein could be digested to a single species, which was essentially deglycoslylated, some residual sugar remained as assessed using an immunoblot kit for detecting glycoproteins from Bio-Rad. This deglycosylated s-erbB1dom3 was however unstable and precipitated during purification attempts. No further work on producing large quantities of the subdomains 1 and 3 of s-erbB receptors has been conducted.

The *Drosophila* expression system has been used to express the ectodomains of erbB1, erbB2 and erbB4. In this system stable Sneider 2 (S2) cell lines are generated for each s-erbB and the expression of protein is induced with copper sulfate. Histidine tags are again used to aid the purification that is essentially identical to that used for the baculovirus expressed proteins. For s-erbB2 and s-erbB4 the yields are substantially higher than could be obtained from Sf9 cells; 10 and 50 mg/L respectively. Expression of s-erbB1 is lower. Attempts are under way to generate a s-erbB1 stable cell line that will express the protein at higher levels. In all characterizations the S2 cell expressed proteins appear to behave identically to those expressed in Sf9 cells.

### *Task 2.* Obtain purified ligands (months 1 - 18)

When considering the EGF-like domains only, there are at least 12 different erbB ligands that need to be compared (Figure 3).

**EGF agonists: activate erbB1 (EGFR)**

```
EGF      NSDSE○PLSHDGY○LHDGV○MYIEALDKYA...○N○VVGYIGER○QYRDLKWWE
TGFα     SHFND○PDSHTQF○FH.GT○RFLVQEDKPA...○V○HSGYVGAR○EHADLLAVV
AR       KKKNP○NAEFQNF○IH.GE○KYIEHLEAVT...○K○QQEYFGER○GEKSMKTHS
```

**Neuregulins: activate erbB3/4**

```
NRG1α    SHLVK○AEKEKTF○VNGGE○FMVKDLSNPSRYL○K○QPGFTGAR○TENVPMKVQ
NRG1β    SHLVK○AEKEKTF○VNGGE○FMVKDLSNPSRYL○K○PNEFTGDR○QNYVMASFY
NRG2β    GHARK○NETAKSY○VNGGV○YYIEGINQLS...○K○PNGFFGQR○LEKLPLRLY
NRG2α    GHARK○NETAKSY○VNGGV○YYIEGINQLS...○K○PVGYTGDR○QQFAMVNFS
NRG3     EHFKP○RDKDLAY○LNDGE○FVIETLTG.SHKH○R○KEGYQGVR○DQFLPKTDS
NRG4     DHEQP○GPRHRSF○LNGGI○YVIPTIPSPF...○R○IENYTGAR○EEVFLPSSS
```

**Bi-specific: activate erbB1 and erbB4**

```
BTC      GHFSR○PKQYKHY○IK.GR○RFVVAEQTPS...○V○DEGYIGAR○ERVDLFYLR
HB-EGF   KKRDP○LRKYKDF○IH.GE○KYVKELRAPS...○I○HPGYHGER○HGLSLPVEN
EPR      VSITK○SSDMNGY○LH.GQ○IYLVDMSQNY...○R○EVGYTGVR○EHFFLTVHQ
```

**Figure 3** **EGF-like domains of erbB ligands.**
EGF, epidermal growth factor; TGFα, transforming growth factor α; AR, amphiregulin; NRG, neuregulin; BTC, betacellulin; HB-EGF, heparin binding-EGF; EPR, epiregulin.

For our initial analysis of homo- and hetero dimerization of s-erbBs[11], we used commercially available EGF and NRG1$\beta_1$. High quality s-erbB1/EGF crystals can be grown using this EGF purchased from Intergen, which is relatively inexpensive. To broaden the scope of out ligand binding analysis we are now also expressing ligands in *Drosophila* S2 cells. We have cell lines to express NRG2α, NRG2β, NRG3 and NRG4 (kindly provided by David Riese, Purdue University). We are in the process of generating cell lines to make additional ligands. C-terminal histadine tags on the ligands aid in purification on a Ni-NTA column as for the s-erbBs. Fractions eluted from a Ni-NTA column that contain the ligand are loaded directly onto a C-18 reverse phase column (Vydac) in 0.1% trifluroacetic acid (TFA) and eluted with a gradient of acetonitrile/0.1% TFA. Fractions containing the protein are lyophilized, resuspended in 50 mM Hepes pH 8.0, 150 mM NaCl, and further purified by size exclusion chromatography on a Superose 12 column (Pharmacia).

***Task 3.*** **Characterize binding of ligands to isolated domains 1 and 3 (months 6 - 12)**

Given our success in generating the full length ectodomains and a variety of ligands, we have chosen to characterize the ligand binding to the entire ectodomains rather than to the isolated ligand binding domains. Initially analysis of ligand binding has been published[11]. Further analysis is on going as new ligand preparations are generated.

***Task 4.*** **Obtain diffraction quality crystals of s-erbB1/EGF complex (months 7 - 18)**

Diffraction quality crystals of the s-erbB1/EGF complex were grown as follows. Purified s-erbB1 was buffer exchanged into 5mM Hepes, 25 mM NaCl, pH 8.0, and concentrated to 30 mg/ml, using a Centricon 30 (Amicon). A 1.2 fold excess of EGF was added to give final receptor concentration of 250 μM. Standard approaches were used to survey for conditions under which s-erbB1 and s-erbB1/EGF complex might crystallize. These included the use of both sparse matrix and grid screening. Small needle like crystals were observed for the s-erbB1/EGF (1.2 fold molar excess of EGF) complex in several PEG/low pH conditions in an early screen. Under the same conditions no crystals formed for the protein alone. The presence of the EGF in the crystal was confirmed by SDS-PAGE analysis of washed crystals (Figure 4). The EGF can clearly be seen and comparison with prepared standard samples indicates that the ligand to receptor stochiometry in the crystal is approximately 1:1.

Subsequent optimization of the crystallization conditions produced much larger crystals, suitable to test the diffraction quality. The best crystals grow from 10 - 15 % PEG 8000 at pH 6.0 to dimensions of about 0.5 x 0.2 x 0.08 mm. To test diffraction, crystals were transferred to a solution containing the same components as the reservoir plus 15% glycerol as cryoprotectant and frozen directly in liquid nitrogen in a nylon loop. Good crystals diffract to better than 3.5 Å on a MAR image plate detector using double mirror-monochromated/focused Cu $K_\alpha$ X-rays from a Siemens generator. The crystals are of space group C2, with a = 118 Å, b = 103 Å, c = 100 Å, β = 119° and one half dimer per asymmetric unit.

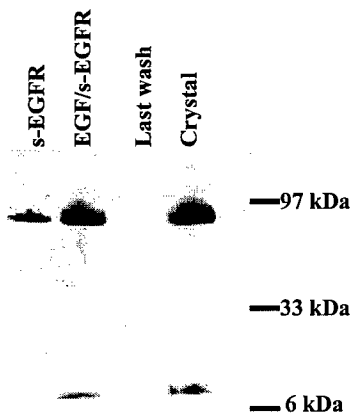


**Figure 4. Crystals contain s-erbB and EGF.**

Silver-stained 8-25% PhastGel of EGF/s-erbB1showing that both EGF and s-erbB1 are present in the crystals. No protein was extracted in the last wash of the crystals. However, after dissolving the washed crystals in water, and subjecting to SDS-PAGE, the relative intensities of the s-erbB1 and EGF bands are similar to that seen when a 1:1 EGF:s-erbB1 complex is run on the gel (lane 2).

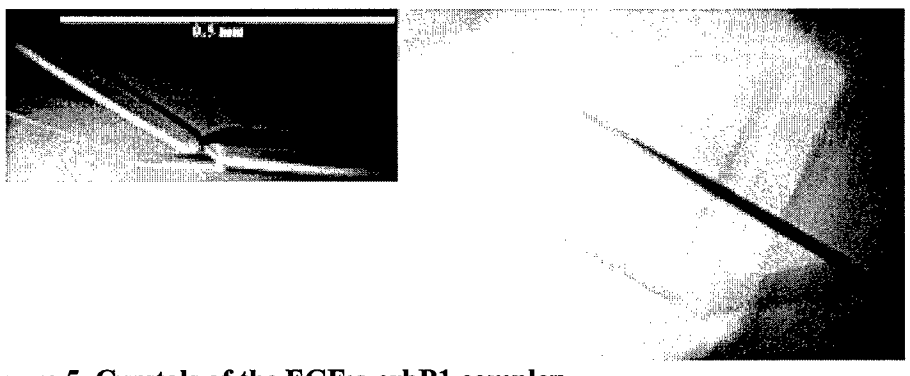


**Figure 5 Crystals of the EGF:s-erbB1 complex.**
The white bar represents 0.5 mm. Both panels are at the same magnification

There is variability in the diffraction quality of the crystals and many have to be tested to find a strong single, crystal. Seeding has been successfully applied and improves reproducibility and quality of the crystals. This project represents a key component in several beam time applications at both the National Synchrotron Light Source at Brookhaven National Laboratories and the Cornell High Energy Synchrotron Source (CHESS).

## *Task 5.* Solve the X-ray crystal structure (months 18 - 30)

(i) Collect high resolution native data (months 18 - 24)

A complete native data set to 2.8 Å was colleted on an unstabilized frozen crystal at the National Synchrotron Light Source (NSLS) beam line X25 operating at 1.0Å with a Brandeis 4-module CCD-Based detector (B4) Data were processed using DENZO and SCALEPACK[12]. A summary of the data collection statistics is given in Table 1.

| Resolution Shells | | Unique Reflections | Completeness | $R_{sym}$* | I/σ |
|---|---|---|---|---|---|
| 50.00 | 5.60 | 3122 | 91.9 | 0.086 | 14.3 |
| 5.60 | 4.44 | 3249 | 97.5 | 0.058 | 19.3 |
| 4.44 | 3.88 | 3320 | 98.6 | 0.071 | 17.8 |
| 3.88 | 3.53 | 3287 | 99.2 | 0.092 | 14.3 |
| 3.53 | 3.27 | 3294 | 99.3 | 0.138 | 10.4 |
| 3.27 | 3.08 | 3313 | 99.6 | 0.277 | 6.4 |
| 3.08 | 2.93 | 3271 | 99.7 | 0.443 | 3.7 |
| 2.93 | 2.80 | 3250 | 97.0 | 0.481 | 2.3 |
| All reflections | | 26106 | 97.8 | 0.087 | 14.3 |

**Table 1** Data collection statistics for Native data collected at NSLS X25.
*$R_{sym} = \Sigma_{h'} |I_h - I_{h'}| / \Sigma_{h'} I_{h'}$, where $|I_h - I_{h'}|$ is the absolute deviation of a reflection $I_{h'}$ from the average ($I_h$) of its symmetry and Friedel equivalents

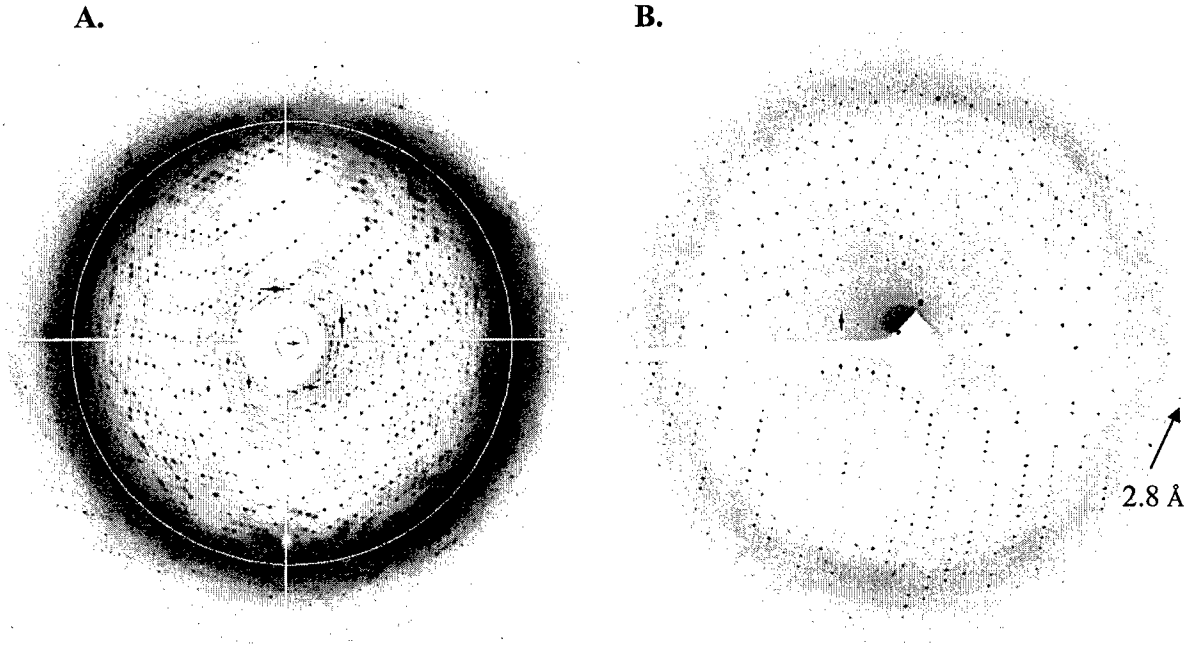


**Figure 6**
Oscillation images for EGF:s-erbB1 crystals. A. Image collected at CHESS beamline F1, using the Q4 CCD detector, with a 0.5° oscillation. Circles are shown at resolutions, 11, 5.3, 3.5 and 2.65 Å. B. Image collected at NSLS beamline X25, using the B4 CCD detector, with a 0.5° oscillation.

While the data extend to 2.8 Å the quality of the high resolution data is poor. Attempts have been made to obtain better high resolution data. At CHESS F1 beam line a crystal was tested that diffracted to beyond 2.6 Å. However the diffraction decayed during the course of the data collection and the final data set was weaker than that above – although of better quality to 3.0 Å.

(ii) Search for heavy atom derivative (months 18 – 30) and

(iii) Collect heavy atom derivative data (months 18 - 30)

The phase information required to calculate an electron density map, from which the structure is deduced, will be obtained by the method of multiple isomorphous replacement (MIR). In this method the positions of heavy atoms in derivatives of the crystal are used to solve the phase problem. The conventional method for obtaining heavy atom derivative is to soak the crystal in solutions containing a variety of different heavy metal ions. These ions can interact with accessible functional groups in the protein crystal such as those on methionines and histidines. For large proteins a common problem with this strategy is that too many heavy atoms bind weakly to the protein. An alternative strategy that can be used in such cases is to prereact the growth factor ligand with a heavy atom that will form a stable complex with the protein.

The first stage in initiating a heavy atom derivative search is to ensure that a crystal soaked in stabilizer without any heavy atom added is isomorphous with previously collected native data. Partial data sets have been collected on home sources for a number of different stabilized crystals. The stabilized crystals were non-isomorphous with the crystal frozen after only brief exposure to the cryoprotectant (as used to collect the data at NSLS X25). This problem has been resolved. A complete data set was collected at NSLS beam line X12B on a crystal stabilized for 24 hours. Data were collected at 1.0Å, with a Area Detector Systems Corporation Quantum 4 CCD detector (Q4), and were processed with MOSFLM[13] and CCP4[14,15]. Statistics for the data set are presented in Table 2.

The quality of the data from this crystal is improved compared to the data collected at beam line X25, although the overall intensity is a little weaker.

| Resolution Shells | | Unique Reflections | Completeness | $R_{sym}$ | I/σ |
|---|---|---|---|---|---|
| 50.00 | 6.26 | 2293 | 95.5 | 0.038 | 15.2 |
| 6.26 | 5.11 | 1926 | 97.0 | 0.038 | 15.6 |
| 5.11 | 4.43 | 2290 | 97.8 | 0.039 | 14.8 |
| 4.43 | 3.96 | 2601 | 98.0 | 0.044 | 14.1 |
| 3.96 | 3.61 | 2879 | 98.5 | 0.064 | 9.3 |
| 3.61 | 3.35 | 3157 | 98.7 | 0.089 | 7.8 |
| 3.35 | 3.13 | 3361 | 98.9 | 0.142 | 5.2 |
| 3.13 | 2.95 | 3602 | 99.1 | 0.254 | 2.9 |
| 2.95 | 2.80 | 3814 | 99.2 | 0.411 | 1.8 |
| All reflections | | 25923 | 98.2 | 0.057 | 9.5 |

**Table 2.** Data collection statistics for native data from a stabilized crystal collected at NSLS X12B.

Three methods are being explored to determine the phase information required to solve the structure. For discussion these methods will be considered separately, although information from each approach may be combined in producing the final phases to calculate an interpretable electron density map.

(a) The use of multiple isomorphous replacement (MIR) is being explored. In this method the positions of heavy atoms in multiple different derivatives of the crystal are used to solve the phase problem. The conventional method for obtaining heavy atom derivative is to soak the crystal in solutions containing a variety of different heavy metal ions. These ions can interact with accessible functional groups in the protein crystal such as those on methionines and histidines.

A number of potential heavy atom derivatives are currently being analyzed. This analysis is greatly aided by the new native data that we collected at X12B. Table 2 shows details some of the data that we are analyzing and the merging R-factor to the X12B data ($R_{iso}$).

| Heavy Atom | Soak Condition | X-ray Source | Resolution Limit | Completeness (multiplicity) | I/σ (mean) | $R_{sym}$ | $R_{iso}$ |
|---|---|---|---|---|---|---|---|
| PIP (Pt) | 1 mM 18 h | Home | 3.8 Å | 95 % (3.5) | 15.6 | 0.09 | 16.6 % |
| PIP (Pt) | 1 mM 18 h | X12B λ=0.9Å | 3.5 Å | 55 % (2.0) | 12.2 | 0.06 | 16.7 % |
| $K_2PtCl_4$ | 1 mM 15 h | Home | 3.8 Å | 52 % (2.8) | 10.5 | 0.09 | 18.8 % |
| $KAuI_4$ | 5 mM 24 h | Home | 3.2 Å | 91 % (2.8) | 17.6 | 0.06 | 12.6 % |
| $UO_2SO_4$ | 1 mM 18 h | Home | 3.8 Å | 61 % (2.2) | 12.7 | 0.09 | 13.8 % |

**Table 3** Data collected for EGF:s-erbB1 crystals soaked with different heavy-atom compounds. PIP, di-μ-iodobis(ethylenediamine)diplatinum dinitrate; $UO_2SO_4$, uranyl sulfate

We have tried exhaustively to identify heavy atom sites in these derivatives by analyzing isomorphous and anomalous difference Patterson maps, using the programs MLPHARE[16] and SOLVE[17]. So far we have been unable to obtain a clear solution for any of these soaked crystals

(b) Recent developments open the possibility that we may be able to solve the structure using the multiwavelength anomalous diffraction (MAD) phasing method[18,19]. In this method data from a single crystal containing several anomalous scatterers (e.g. selenium atoms) are collected at several different wavelengths. We will generate crystals of a selenomethione derivative of s-erbB1 in complex with wild type EGF (EGF has no methionines). There are 10 methionines in the 621 amino acids of mature s-erbB1, and alignment with the IGF1-R structure (see below) indicates that at least half of these will be in well-ordered regions. If high-efficiency substitution with selenomethionine can be achieved at these positions, there is a significant chance that the EGF:s-erbB1 complex can be solved using MAD phasing.

The selenomethionine (Se-Met) is incorporated biosynthetically by expressing the protein in cells that have Se-Met as the only source of methionine. We have performed this many times to produce Se-Met proteins in *E. coli*. The application of this method to protein expressed in insect cells is relatively new[30-33]. Bellizzi *et al* have modified and optimized the method for expression of secreted proteins and have solved the structure of an insect cell expressed secreted protein using the MAD method[22,23]. We have expressed a selenomethionine analogue of s-erbB1 (Figure 7). Sf21 cells are grown in standard serum free medium and infected with recombinant virus as usual. Thirty six hours after infection with virus the cells are collected by centrifugation, and transferred to a medium containing no methionine. Cells are incubated in this medium for 4 hours to allow methionine to be cleared from the cells. Following this clearing period cells transferred to methionine minus medium containing 50 mg/L selenomethionine. After a further 3 days, the conditioned media is harvested and the Se-Met erbB1 purified as for the wild type protein. Pilot analysis indicated that the expression level is not compromised by the use of Se-Met (Figure 7). Large scale preparations of this Se-Met protein have been conducted. When subjected to quantitative amino acid analysis s-erbB1 grown in this way was found to be only about 50 % substituted Se-Met for Met. We have collected single wave length experiments. While the Se can be seen in the crystals observing the x-ray absorption spectrum, no solution to the anomalous difference Patterson maps has been identified. Future work will be directed toward increasing the Se-Met incorporation rate (by increasing the clearing period), and obtaining better quality data, where the small anomalous differences will be more accurately measured.

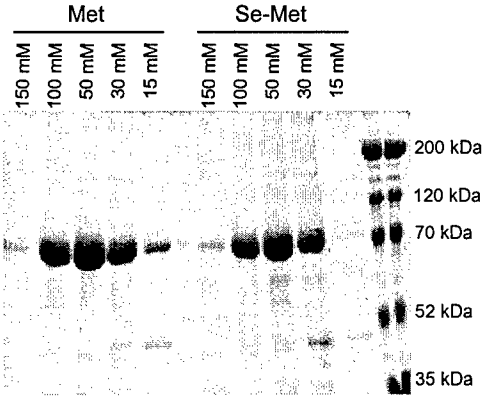


**Figure 7**

Coomassie stained SDS PAGE of Met and Se-Met s-erbB1. Fractions were eluted from a Ni-NTA column with increasing concentrations of imidazole. Cultures used to express these proteins were treated in exactly the same way except, for samples on the left, 50 mg/L methionine were added in the final incubation period, whereas for samples on the right, 50 mg/L for selenomethionine were used

(c) The final method that may be useful in gaining phasing information is molecular replacement (MR). The 2.6 Å resolution x-ray crystal structure of the first three extracellular domains (ECD) of the insulin-like growth factor type 1 receptor (IGF-1R) has been solved[24] and the coordinates have now become available. The first three subdomains of the IGF1-R ECD are closely related to the first three (of four) subdomains in s-erbB1. Subdomains 1 and 3 of s-erbB1, which share 31% identity, align well with subdomains L1 and L2 of the IGF1-R ECD. Subdomains L1 and L2 of IGF1-R share 22% identity, and the $C_\alpha$ positions of their respective β-helix structures superimpose with a root-mean-squared deviation of 1.6 Å. The sequences of IGF1-R L1 and L2 align better with D1 and D3 of s-erbB1 than they do with one another (Figure 8), suggesting that s-erbB1 subdomains D1 and D3 will have similar β-helix structures.

```
IGF1R L1   EICGP...GIDIRN.........DYQQLKRLENCTVIEGYLHILLISKA.........EDYRS
EGFR D1    KVCQ....GTSNKLTQLGTFEDHFLSLQRMFNNCEVVLGNLEITYVQRN............YD
EGFR D3    KVCN....GIGIGEFKDSLSI..NATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQE
IGF1R L2   KVCEEEKKTKTID..........SVTSAQMLQGCTIFKGNLLINIRRGN.......NIASELE

IGF1R L1   YRFPKLT.VITEYLLLFRVAGLESLGDLFPNLTVIRGWKLFY.NYALVIFEMTNLKDIGL...
EGFR D1    LSFLKTIQEVAGYVLIALNT.VERI..PLENLQIIRGNMYYENSYALAVLSNYDANKTGLKEL
EGFR D3    LDILKTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSL.NITSLGL...
IGF1R L2   NFMGLIE.VVTGYVKIRHSHALVSLS.FLKNLRLILGEEQLEGNYSFYVLDNQNLQQLWDW..

IGF1R L1   ..YNLRNITRGAIRIEKNADLCYLSTVDWSLILD...AVSNNYIV..GNKPPKECGD
EGFR D1    PMRNLQEILHGAVRFSNNPALCNVESIQWRDIVS...SDFLSNMSMDFQNHLGSCQK
EGFR D3    ..RSLKEISDGDVIISGNKNLCYANTINWKKLFGT..SGQKTKII..SNRGENSCKA
IGF1R L2   DHRNL.TIKAGKMYFAFNPKLCVSEIYRMEEVTGTKGRQSKGDINTRNNGERASCES
```

**Figure 8** Alignment of the ligand-binding subdomains of EGF receptor (D1 and D3) with the equivalent subdomains in the IGF1 receptor ECD (L1 and L2). Sequence identities in the alignments are: $L1_{IGF1\text{-}R}/L2_{IGF1\text{-}R}$, 22%; $D1_{erbB1}/D3_{erbB1}$, 31%; $L1_{IGF1\text{-}R}/D1_{erbB1}$, 25%; $L1_{IGF1\text{-}R}/D3_{erbB1}$, 29%; $L2_{IGF1\text{-}R}/D1_{erbB1}$, 26%; $L2_{IGF1\text{-}R}/D3_{erbB1}$, 25%.

Molecular replacement has be attempted using L1, L2, and L1 plus L2 as search models, using AmoRe[25] and the Patterson correlation technique in XPLOR[26]. Initial search models were generated by trimming all non-conserved side-chains to the β-carbon. Thus far no clear solution has been obtained. Since appropriately trimmed L1 and L2 search models together correspond to only around 30% of the scattering power of our EGF:s-erbB1 crystal asymmetric unit (assuming 59% solvent content), it is not surprising that the a solution has not been obtained easily. A theoretical model proposed for s-erbB1 on the basis of the x-ray crystal structure of IGF-1R[27] has also been used as a molecular replacement search model. Again no solution was obtained.

*Task 6.* Build the atomic model and refine the structure (months 30 - 36)

- Build model into the experimental electron density map (months 30 - 32)
- Refine the model to high resolution (months 32 - 36)

Since no phases were obtained it has not been possible to proceed with this task. Further grant support (see below) has been obtained that will allow continuation of this project.

Since there has been less progress than hoped in solving the s-erbB1/EGF structure, we have attempted to obtain crystals of other s-erbBs. Recently we obtained crystals of s-erbB2 (Figure 9). Preliminary analysis on home sources of clusters of crystal indicate that diffraction to better than 3 Å is expected. Work is in progress to obtain good quality single crystals.

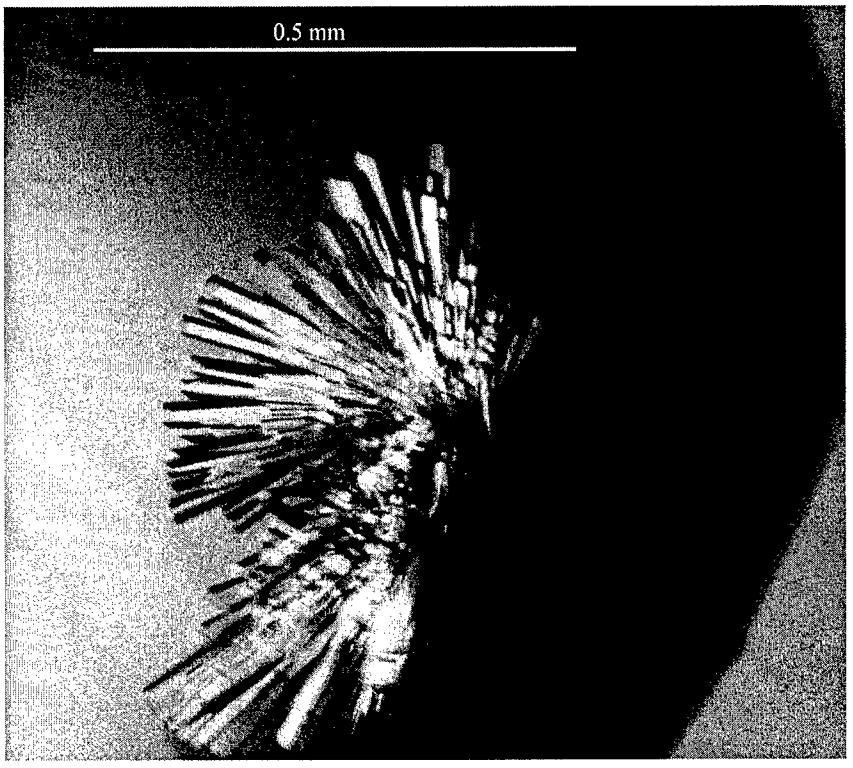


**Figure 9 Crystals of the s-erbB2.** The white bar represents 0.5 mm

## Key Research Accomplishments

- Well diffracting crystals of s-erbB1/EGF complex.
- 2.8 Å native data for the s-erbB1/EGF complex.
- Substantial progress toward solving the structure of the s-erbB1/EGF complex
- Crystals of s-erbB2

## Reportable Outcomes

Manuscripts:

Ferguson, K.M., Darling, P.J., Macatee, T.L., Mohan, M. and Lemmon, M.A. (2000) Extracellular Domains Drive Homo- but not Hetero-Dimerization of ErbB Receptors. *EMBO J.* **19,** 4632-4643.

Ferguson, K.M., Kavran, J.M., Sankaran, V.G., Fournier, E., Isakoff, S.J., Skolnik, E.Y., and Lemmon, M.A. (2000). Structural basis for discrimination of 3-phosphoinositides by pleckstrin homology (PH) domains. *Molecular Cell,* **6,** 373-384.

Lemmon, M.A. and Ferguson K.M. (2000) Membrane-targeting by pleckstrin homology (PH) domains. *Biochem. J.* **350,** 1-18.

Lemmon, M.A., and Ferguson, K.M. (2000) Pleckstrin Homology domains: phosphoinositide-regulated membrane tethers. in Cockcroft, S. (Ed.) *Biology of Phosphoinositides: Frontiers in Molecular Biology* **27,** 131-165. Oxford University Press.

Abstracts:

Ferguson, K.M., and Lemmon, M.A. (2000) Structural Analysis of Ligand Binding by the Epidermal Growth Factor Receptor Family of Tyrosine Kinases. Era of Hope Meeting for the Department of Defense Breast Cancer Program.

Ferguson, K.M., Macatee, T.L., and Lemmon, M.A. (2000) Extracellular Domains are Sufficient for Ligand Induced Homo- but not Hetero-dimerization of erbB Receptors. . Era of Hope Meeting for the Department of Defense Breast Cancer Program.

Ferguson, K.M., and Lemmon, M.A. (2001) Structural Analysis of Ligand Induced erbB Receptor Dimerization. Career Awardee Meeting, Burroughs Wellcome Fund.

Presentations:

March 2001 Seminar Kimmel Cancer Center, Thomas Jefferson University, Philadelphia PA.
"Structural views of molecular recognition in growth factor signaling"

June 2001 Seminar Department of Cellular & Molecular Physiology, Yale University, New Haven, CT.
"Recognition of lipid second messengers by pleckstrin homology (PH) domains"

Grant Support

| | | |
|---|---|---|
| NIH Grant | R21-CA87182-01 (Lemmon) | 7/1/00 – 6/30/03 |
| NIH/NCI | | $ 75,000 p.a. |

Based upon the results obtained by the PI during the first and second years of this award, Mark A. Lemmon, the PI's mentor, has applied for and been granted an NIH Insight Award to complete the structure of the s- erbB1/EGF complex. This grant will provide salary for a technical assistant and supplies.

Howard Temin Award K01-CA92246-01 (Ferguson) 7/01/01 – 6/30/06
NCI $ 126,000 in first year

Based upon the results obtained by the PI during this award, the PI has applied for and been granted an NCI career development award, to expand the structural analysis to s-erbB2. The first year of this grant will provide salary for the PI upon termination of the DOD BCRP award. Upon obtaining a faculty position this grant will also provide additional funds for technical assistant.

Career Award in the Biomedical Science (Ferguson) 9/1/01 – 8/31/06
Burrough Wellcome Fund $ 60,000 in first year

Based upon the success in crystallizing s-erbB1, the PI has applied for and been granted a career development award to further expand the biochemical and structural analysis of s-erbB3 and s-erbB4 and their ligands – the neuregulins. This award provides money for supplies. Upon obtaining a faculty position this award will increase to $ 120,000 p.a

Employment Opportunities

The PI has been promoted to a research associate at the University of Pennsylvania.

The PI has begun to seek a faculty position and has had interviews for tenure track positions at the Kimmel Cancer Center, Thomas Jefferson University; Department of Cellular & Molecular Physiology, Yale University, and Department of Physiology, University of Pennsylvania.

## Conclusions

The specific aims of this proposal were as follows:

- Determine the structure of the ligand binding, domain 3, from erbB1 in complex with EGF.
- Characterize the determinants of ligand specificity of domains 1 and 3 for each of the erbB receptors.
- Determine the structure of the EGF induced s-erbB1 homodimer
- Determine the structure a ligand induced heterodimer of s-erbB2 and one of the other s-erbB receptors.

The first of these two aims was superceded by our rapid success in obtaining well diffracting crystals of the s-erbB1/EGF complex. It has proven more informative to analyze the ligand binding characteristics of the intact ectodomains and the same question as the second initial specific aim are being addressed with these proteins. Some of these specificity studies have already been published[11], and others are on going as we develop expression systems for additional ligands.

The bulk of the effort during the award period was devoted to the third specific aim – determination of the structure of the s-erbB1/EGF complex. As outlined in this report the structure has not yet been solved, but substantial progress has been made towards this aim. The s-erbB1/EGF structure will provide the first view of the interactions that stabilize active erbB receptor dimers. In addition to answering key questions about the mechanism of erbB receptor activation, the structure of the s-erbB1/EGF dimer will provide a critical starting point for investigating the complex array of receptor/ligand interactions seen in the larger erbB receptor system.

We have recently begun working on the fourth specific aim, obtaining crystals of isolated s-erbB2. The PI plan to solve the structure of the extracellular domain of erbB2, the member of the family that is most straight forwardly implicated in cancer. This structure will provide insight into why certain anti-erbB2 antibodies (such as Herceptin) are anti-proliferative, yet others are not, and will aid the design of improved drugs.

We have been unable to complete all the studies that we have initially proposed. This is not unusual for crystallographic projects where major obstacles (obtaining crystals, obtaining phase information) can delay a projects progress. We have been unable to obtain the phase information that we need to solve the s-erbB1/EGF complex. However we have secured funding to continue with this project, both while the PI is still a postdoc in Mark Lemmon's laboratory and when the PI moves on to an independent position.

## References


1. Ullrich, A. & Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61,** 203-212.
2. Hynes, N.E. & Stern, D.F. (1994) The biology of *erbB-2/neu/HER-2* and its role in cancer. *Biochim. Biophys. Acta.* **1198,** 165-184.
3. Slamon, D.J., Goldolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. & Press, M.F. (1989) Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. *Science* **244,** 707-712.
4. Dougall, W.C., Qian, X., Peterson, N.C., Miller, M.J., Samanta, A. & Greene, M.I. (1994) The neu oncogene: Signal transduction pathways, transformation mechanisms and evolving therapies. *Oncogene* **9,** 2109-2123.
5. Bacus, S.S., Zelnick, C.R., Plowman, G. & Yarden, Y. (1994) Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. *Am. J. Clin. Pathol.* **102,** S13-24.
6. Lemmon, M.A. & Schlessinger, J. (1994) Regulation of signals transduction and signal diversity by receptor oligomerization. *Trends Bioc. Sci.* **19,** 459-463.
7. Lemmon, M.A. & Schlessinger, J. (1997) Transmembrane signaling by receptor oligomerization. In *Methods in Molecular Biology , Vol 84: Transmembrane Signaling Protocols* (Bar-Sagi, D., ed.) Humana Press Inc., Totowana, NJ, pp 31-53.
8. Pawson, T. (1995) Protein modules and signaling networks. *Nature* **373,** 573-580.
9. Riese, D.J. 2nd, and Stern, D.F. (1998) Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays* **20,** 41-48.
10. Lemmon, M.A., Bu, Z., Ladbury, J.E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D.M. & Schlessinger, J. (1997). Two EGF molecules contribute additively to stabilization of the EGFR dimer. *EMBO J.* **16,** 281-294..
11. Ferguson, K.M., Darling, P.J., Macatee, T.L., Mohan, M. and Lemmon, M.A. (2000) Extracellular Domains Drive Homo- but not Hetero-Dimerization of ErbB Receptors. *EMBO J.* **19,** 4632-4643.
12. Otwinowski, Z. & Minor, W. (1997) Processing of x-ray diffraction data collection in oscillation mode. *Methods Enzymol.* **276** 307-326
13. Leslie, A.G.W. (1992) Joint CCP4 & ESF-EAMCB Newsletter on Protein Crystallography, No. 26.
14. Collaborative Computational Project, Number 4 (1994) "The CCP4 Suite: Programs for Protein Crystallography". *Acta Cryst.* **D50,** 760-763
15. P.R.Evans (1993) "Data reduction", Proceedings of CCP4 Study Weekend on Data Collection & Processing, pp 114-122
16. Otwinowski, Z. (1991). ML-PHARE CCP4 Proc. 80-88 (Daresbury Laboratory, Warrington, UK).
17. Terwilliger, T.C., and Berendzen, J. (1996) Correlated phasing of multiple isomorphous replacement data. *Acta Cryst.* **D52,** 749-757.
18. Hendrickson, W.A., Smith, J.L., Phizackerley, R.P., and Merritt, E.A. (1988) Crystallographic structure analysis of lamprey hemoglobin from anomalous dispersion of synchrotron radiation. *Proteins: Struct., Funct., & Genet.* **4,** 77-88.
19. Hendrickson, W.A., Pahler, A., Smith, J.L., Satow, Y., Merritt, E.A., and Phizackerley, R.P. (1989) Crystal structure of core streptavidin determined from multiwavelength anomalous diffraction of synchrotron radiation. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 2190-2194.
20. Chen, W., and Bahl, O.P. (1991) Recombinant carbohydrate and selenomethionyl variants of human choriogonadotropin. *J. Biol. Chem.* **266,** 8192-8197.
21. Chen, W., and Bahl, O.P. (1991) Selenomethionyl analog of recombinant human choriogonadotropin. *J. Biol. Chem.* **266,** 9355-9358.
22. Bellizzi, J.J., Widom, J., Kemp, C.W., Clardy J (1999) Producing selenomethionine-labeled proteins with a baculovirus expression vector system. *Structure Fold Des.* **7,** R263-7.
23.. Bellizzi, J.J., Widom, J., Kemp, C., Lu, J.Y., Das, A.K., Hofmann, S.L., Clardy J. (2000) The crystal structure of palmitoyl protein thioesterase 1 and the molecular basis of infantile neuronal ceroid lipofuscinosis. *Proc Natl Acad Sci (U S A)* **97,** 4573-4578.
24. Garrett, T.P.J, McKern, N.M., Lou, M., Frenkel, M.J., Bentley, J.D., Lovrecz, G.O., Elleman, T.C., Cosgrove, L.J. & Ward, C.W. (1998) Crystal structure of the first three domains of the type-1 insulin-like growth factor receptor. *Nature* **394,** 395-399.

25. Navaza, J. (1994) AMoRe: An automated package for molecular replacement. *Acta Cryst. A.* **50,** 157-163.
26. Brünger, A.T. (1992a). XPLOR Version 3.1. A System for X-Ray Crystallography and NMR (New Haven, Connecticut: Yale University Press).
27. Jorissen, R.N., Epa, V.C., Treutlein, H.R., Garrett, T.P., Ward, C.W., Burgess, A.W. (2000) Characterization of a comparative model of the extracellular domain of the epidermal growth factor receptor. *Protein Sci* **9,** 310-324.

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